GENETIC CONTROL OF MULTIPLE FORMS OF TREHALASE IN *NEUROSPORA CRASSA*[1]

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TREHALOSE, an α,α-diglucoside, is the most widely distributed disaccharide in fungi and insects, and is also found in a variety of other organisms. In fungi it probably serves as a source of energy during germination, and the utilization of trehalose may be associated with spore formation as well (SUSSMAN 1961, 1969). The importance of trehalose in the metabolism of Neurospora and other organisms has been studied by several investigators (CLEGG 1965; FAIRBAIRN and PASSEY 1957; SUSSMAN 1969; WYATT and KALF 1956, 1957). Except for a few reports on procaryotes, very little is known concerning the genetics and the control of trehalase, the hydrolytic enzyme which is responsible for the cleavage of the α–α bond of trehalose. Recently, we have found two forms of trehalase and mutants of Neurospora which lack such activity. This paper is a report on the mechanism of the inheritance of the different forms of the enzyme.

MATERIALS AND METHODS

The exotic strains used were kindly provided by Dr. DAVID PERKINS of Stanford University, and strain 4–121A was obtained from one originally provided by Dr. NORMAN HOROWITZ. Other strains were obtained from the Fungal Genetics Stock Center (FGSC) and are listed below:

Locus	Allele (isolation #)	FGSC #	Description
fr	B110	102	colonial
inos	89601	497, 498	requires inositol
me-6	35809	301	requires methionine
pe	Y8743m	37	peach-colored conidia
fld	p628	554	no macroconidia, few or no microconidia
pe. fl	Y8743m	569	abundant microconidia
pab-2. ylo-1	Y30539y	225	aconidial, peach-colored

Preparation of cultures: Cultures were grown in 125 ml Erlenmeyer flasks containing 30 ml minimal medium which consisted of 2% VOGEL's (1964) salts (v/v), 2% sucrose (w/v), and supplements when needed. After 5 days of growth the mycelium was harvested over a Büchner funnel and washed with approximately 200 ml of distilled water. Then the mycelial mat was washed with approximately 200 ml of acetone and dried at room temperature.

Preparation of enzyme extracts: Mycelial mats were ground with cold acetone (4°C) in a Sorval Omnimixer and the powder was harvested on a Büchner funnel, air-dried, and stored at —20°C until use. Approximately 100 mg of acetone-dried mycelial powder was suspended in 2 ml of phosphate buffer (0.05 M, pH 5.6), hereafter called normal buffer, and stored in the cold (4°C) overnight, after which the suspension was centrifuged at 10,000 × *g* for 30 min and the

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supernatant fluid collected. Extracts of dilute samples were concentrated by vacuum dialysis in Visking cellulose tubing at 4°C.

Assays: The protein content of extracts was measured as described by LOWRY *et al.* (1951). Trehalase activity was determined by the method of HILL and SUSSMAN (1963).

Electrophoretic studies: Different forms of trehalase were detected by polyacrylamide gel electrophoresis according to the method described by EILERS *et al.* (1964) and METZENBERG (1964). Localization of trehalase on gels was accomplished by coupling trehalase with glucose oxidase in the presence of nitro-blue tetrazolium (nitro-BT), phenazine methosulphate, and trehalose. Glucose oxidase was added to the gels prior to polymerization and the staining solution consisted of 0.5 mg/ml nitro-BT, 0.2 mg/ml phenazine methosulphate, and 1 mg/ml trehalose in normal buffer. Experiments revealed that a 10-fold range (0.4 mg/ml–4.0 mg/ml) in protein concentration of extracts results in no change in trehalase migration.

RESULTS

Fast and slow trehalase: Two distinct forms of trehalase were recognized on the basis of their mobility in polyacrylamide gels. When acetone-dried mycelial powders of strain 89601A were suspended in normal buffer and the soluble fraction used for electrophoresis, a trehalase migrating at *Rf* 0.7 was found. Strain 4–121A yielded a different form of trehalase with an *Rf* of 0.6 under the same conditions (Figure 1). The trehalase from strain 89601A is referred to as the fast trehalase, hereafter designated as F; and that from strain 4–121A is referred to as slow trehalase, hereafter designated as the S form. These two forms of trehalase retain their identity on the gels even upon electrophoresis of an extract composed of partially purified trehalase from strain 89601A mixed with partially purified trehalase from 4–121A (Figure 1).

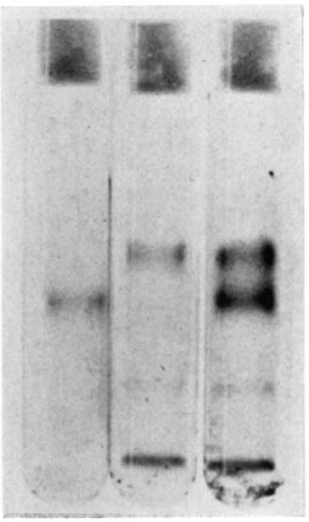

FIGURE 1.—Polyacrylamide gels of fast and slow trehalase. Samples were applied to the top of the gels and migrated towards the anode.

left: fast trehalase of strain 89601A
center: slow trehalase of strain 4–121A
right: *in vitro* mixture of fast and slow trehalase

TABLE 1

Electrophoretic migration of trehalase from the soluble fraction of different strains of Neurospora crassa

Strains	Mating type	Location	Fast	Slow
Stock cultures				
89601 (FGSC#497)	A		X	
89601 (FGSC#498)	a		X	
4-121	A			X
35809 (FGSC#301)	A		X	
Y8743m (FGSC#37)	A		X	
Y8743m (FGSC#569)	a			X
p628 (FGSC#554)	a		X	
Y30539y (FGSC#225)	A		X	
Exotic strains				
Townsville-1	a	Queensland, Australia	X	
Cairns-1c	A	Queensland, Australia	X	
Channapatna-1	A	S. India	X	
Golur-1c	a	S. India	X	
Kadakola-1g	a	S. India	X	
Kalastwadi-1c	a	S. India	X	
Kurubara Shettihally-1f	A	S. India		X
Varkud-1b	a	S. India	X	
Mysore-1e	a	S. India	X	
Lahore-1	A	W. Pakistan	X	X
Besakih-1b	a	Bali, Indonesia	X	
Gianjor-1	A/a	Bali, Indonesia		X
Gianjor-1c	A	Bali, Indonesia	X	
Tampaksiring-1b	a	Bali, Indonesia	X	
Bogor-4f 14	A	Java, Indonesia		X
Bogor-3c	a	Java, Indonesia		X
Bogor-2g	a	Java, Indonesia	X	
Louwi-Malang-1c	A	Java, Indonesia	X	
Obama-1b	a	Kyushu, Japan	X	
Unzen-1	A	Kyushu, Japan		X
Kuala Lumpur-1b	A	Malaya	X	
Kuala Selengor-1	A	Malaya	X	
Klang-6d	A	Malaya	X	
Singapore-1	a	Singapore	X	
Lea f_1-12	A	New Guinea	X	
Rouna-4	a	New Guinea		X
Manila-1c	a	Philippines		X
Manila-8	A	Philippines	X	
Chia-i-1	A	Taiwan	X	
Fu-wei	A	Taiwan		X
Fua-lien-1	A	Taiwan	X	
Kao-shong	a	Taiwan		X
Shing-in	A	Taiwan	X	
Taipei-1g	a	Taiwan	X	

A survey of trehalase from different strains obtained from the Fungal Genetics Stock Center indicated that most possess the F form (Table 1). In addition, the results shown in Table 1 indicate that most of the exotic strains studied show the F form and there is no obvious correlation between different forms of trehalase and their geographical distribution. The coexistence of F and S in some exotic strains may be due either to impure cultures or natural heterokaryosis (DAVIS 1966).

Genetics of fast and slow trehalase: Analysis of randomized and ordered ascospores from crosses between strains having F and S forms of trehalase suggests that there is a 1:1 segregation, indicating that they are controlled by a pair of

TABLE 2

Random-spore analysis of the cross of strain 4–121A (slow form of trehalase) and strain 89601a (fast form of trehalase) of Neurospora crassa

	Parentals		Recombinants		Total
	a, mig^f	*A, mig*^{s}	*a, mig*^{s}	*A, mig*^{f}	
Observed	58	65	29	27	179
Expected*	44.75	44.75	44.75	44.75	179
	$\chi^2 = 25.7$	df = 3	$P < 0.001$		

* Based on independent assortment (i.e., a 1:1:1:1 ratio).

alleles of a single gene which is designated *mig* (Table 2). Due to the association of mating type (mating type is on the left arm of linkage group I) with *mig*, it was predicted that the latter gene is on linkage group I (Table 2). Analysis of crosses between strains having markers on each arm indicated that *mig* is on the right arm of linkage group I. Several three-point crosses were used to estimate map distance and to determine the gene order (Tables 3 and 4). Due to the difficulty in running large numbers of gels, the map distance may not be accurate but the order of *mig* with respect to *al-2* and *me-6* is quite clear (Figure 3). Thus, our data suggest that the distance between *me-6* and *mig* is approximately 8 map units, that between *nic-2* and *mig* is approximately 10 map units, and that between *mig* and *al-2* is approximately 20 map units.

Variants of fast and slow trehalase: Crosses between strains B110a and 4–121A yielded some progeny having trehalase with atypical electrophoretic mobility, either intermediate between F and S, or faster than that of the F form (Figure 2).

TABLE 3

Results of the cross of F and S strains of Neurospora crassa *with markers on linkage group I*

Zygote genotype →				I	II	
				nic-2	*mig*^{f}	*al-2*
				+	*mig*^{s}	+
Progeny classes	Genotypes of progeny			Number of individuals	Total	Percentage
Parental combinations	*nic-2*	*mig*^{f}	*al-2*	143	298	72.68
	+	*mig*^{s}	+	155		
Region I singles	*nic-2*	*mig*^{s}	+	14	30	7.32
	+	*mig*^{f}	*al-2*	16		
Region II singles	*nic-2*	*mig*^{f}	+	20	70	17.07
	+	*mif*^{f}	+	10		
	+	*mig*^{s}	*al-2*	50		
Doubles regions I and II	*nic-2*	*mig*^{s}	*al-2*	2	12	2.93
	+	*mig*^{f}	+	10		
Total				410		

TABLE 4

Ordered ascus analysis of three-point cross between F and S strains of Neurospora crassa

Zygote genotype →	I centromere / centromere	+ / *me-6*	II *mig*^{s} / *mig*^{f}
Number of asci recovered			
Parental tetrads			17
Recombinant tetrads			
Singles, region I			6
Singles, region II			3
Doubles, 4-strand			0
Doubles, 3-strand			0
Doubles, 2-strand			1
Total number of asci isolated			27

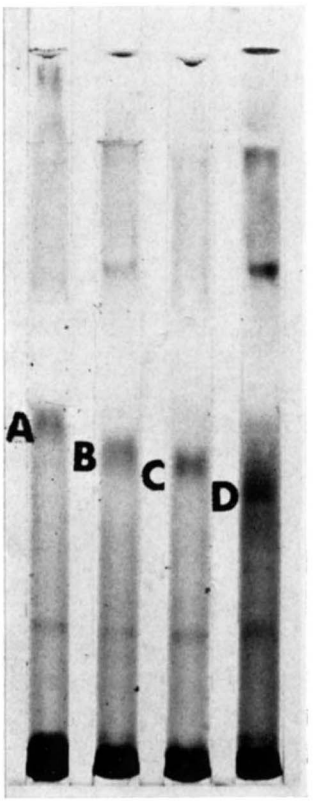

FIGURE 2.—Polyacrylamide gels of "slow" (A), "intermediate" (B), "fast" (C), and "faster" (D) forms of trehalase. Samples were applied to the top of the gels and migrated towards the anode. Bands other than those labeled are nonenzymatic.

TABLE 5

Types of trehalase formed by randomly selected progeny of crosses between strains having different forms of trehalase

Crosses*	Number of progeny			
	Slow	Intermediate	Fast	Faster
Slow × Slow	29	..	..	..
Intermediate × Slow	9	9	..	..
Fast × Fast	..	..	12	..
Fast × Faster	..	..	6	6
Intermediate × Fast	19	23	36	22

* Parents described according to type of trehalase formed.

Furthermore, when the trehalase of B110a was compared to that of standard fast and slow strains, the electrophoretic mobility of the B110a enzyme was found to be faster than that of the standard fast. The inheritance of the intermediate and faster forms produced by progeny from crosses involving B110a was studied by standard crossing procedures, the results of which are presented in Table 5. These results can best be explained in terms of the following hypotheses. The electrophoretic properties of the fast and slow enzymes can be modified by another allelic pair, designed *mod* (*mod*$^+$ and *mod*$^-$). The combination *mig*^{s}*mod*$^-$ produces the slow form and the combination *mig*^{f}*mod*$^-$ the fast form. The combination *mig*^{s}*mod*$^+$ results in the intermediate form and *mig*^{f}*mod*$^+$ the faster form.

DISCUSSION

Many enzymes have been reported to exist in more than one molecular form in the same organism (MARKERT 1968; SCANDALIOS 1968; SHAW 1969) and multiple forms of β-galactosidase (LESTER and BYERS 1965), glutamate dehydrogenase (CODDINGTON, FINCHAM and SUNDARAM 1966), invertase (TREVITHICK and METZENBERG 1964), and other enzymes have been detected in *Neurospora crassa*. Isozymes may arise from (1) association of different numbers of polypeptides, and/or (2) combination of the polypeptides with small molecules having amino, carboxyl or carbohydrate groups, to produce conjugated polypeptide chains with altered properties (MARKERT 1968; SCANDALIOS 1968; SHAW 1969). Study of the inheritance of the two forms of trehalase in Neurospora indicates that they are controlled by a pair of alleles (*mig*^{f} and *mig*^{s}) on linkage group I. The location of the *mig* gene with respect to *nic-2*, *me-6*, and *al-2* is quite clear, but the map distances estimated on the basis of our data are not in agreement with those of DAVIS and DE SERRES (1970), possibly because we were able to analyze only a small population. Alternatively, differences in genetic background may explain the disparity in map distances estimated from different crosses (MITCHELL, 1969), or chromosomal abnormalities such as deletions, inversions, etc. could also account for such discrepancies.

The molecular basis and biological significance of the two forms of trehalase are not yet clear, but the F form seems to have a wider distribution than the

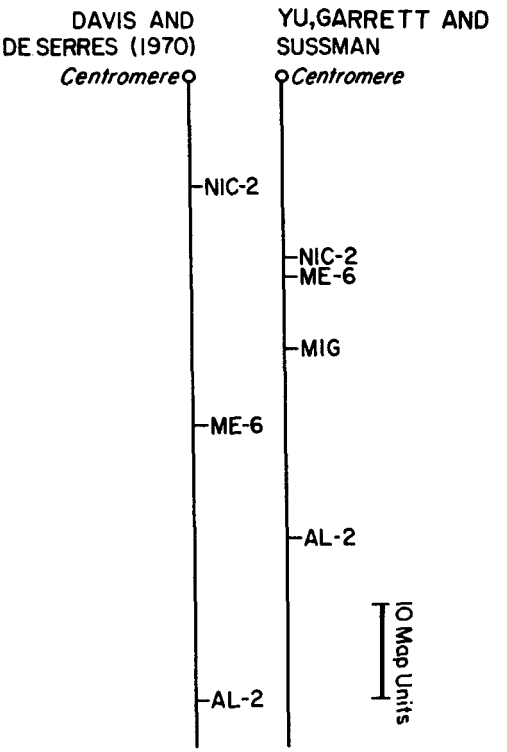

FIGURE 3.—Partial map of linkage group I compiled by DAVIS and DE SERRES (1970) and present authors. All loci on the YU, GARRETT and SUSSMAN map constructed from data in this paper.

S form (Table 1). Therefore, the F form may provide a selective advantage over the S form in some but not all environmental conditions. Trehalaseless mutants have been induced in our laboratory and preliminary studies indicate that the locus for the synthesis of trehalase is also on linkage group I and closely linked to the *mig* gene (SUSSMAN, GARRETT, SARGENT and YU 1971). The relationship between these two genes is being studied, and hopefully such information may provide further insights into the means through which trehalase is controlled.

The genetic nature of the trehalase modifier (*mod*) and its effect on trehalase are not clear at this moment. Such a gene might code for the synthesis of an enzyme responsible for the addition or cleavage of carbohydrate moieties or amino acid side chains of trehalase, thereby affecting its electrophoretic properties. However, it is likely that the *mod* gene is unlinked to *mig*, so at least two genes specify the various isozymes of trehalase. *mig* and *mod* may be two different modifier genes which alter the electrophoretic mobility of trehalase. Alternatively *mig* may be the structural gene for trehalase, the F and S forms being alternative products of alleles at the *mig* locus, and *mod* would then alter their electrophoretic mobility. Our current work is directed towards distinguishing between these and various other hypotheses.

Additional forms of trehalase isolated from cytoplasmic and wall fractions yield different electrophoretic varieties of the enzyme (SUSSMAN. YU and WOOLEY 1971). Inasmuch as the wall fraction of 89601A migrates differently from that of strain 4–121A, several other isozymic forms of trehalase probably exist in Neurospora. These findings are consistent with the conclusion of HILL and SUSSMAN (1963) that there is more than one kind of trehalase in this organism. A similar situation exists for invertase, cytoplasmic and wall forms of which have been shown to exist in Neurospora. (TREVITHICK and METZENBERG 1964).

SUMMARY

Two forms of trehalase with different electrophoretic migration rates were found in the soluble fraction of the mycelium of *Neurospora crassa*. These are controlled by a pair of alleles of a gene designated *mig* on the right arm of linkage group I between *me-6* and *al-2*. The distance between *me-6* and *mig* is about 8 map units, that between *nic-2* and *mig* is about 10 map units, and the distance between *mig* and *al-2* is about 20 map units. The electrophoretic properties of these two forms of trehalase can be modified by another gene designated *mod*, which is not linked to *mig*.

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